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# DNA barcoding and morphological studies reveal two new species of waxcap mushrooms (Hygrophoraceae) in Britain

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## Abstract

Rigorous diagnostics and documentation of fungal species are fundamental to their conservation. During the course of a species-level study of UK waxcap (Hygrophoraceae) diversity, two previously unrecognized species were discovered. We describe *Gliophorus europerplexus* **sp. nov.** and *G. reginae* **sp. nov.**, respectively orange–brown and purple–pink waxcap mushrooms, from nutrient-poor grasslands in Britain. Both share some morphological features with specimens assigned to *Gliophorus* (=*Hygrocybe*) *psittacinus*. However, analysis of sequences of the nuclear ITS DNA barcode region from these and related taxa confirms the phylogenetic distinctness of these lineages. Furthermore, we demonstrated that the holotype of *Hygrophorus perplexus*, a North American species morphologically resembling *G. europerplexus*, is phylogenetically divergent from all our collections. It is likely that further collections of *G. europerplexus* will be revealed by sequencing European material currently filed under *G. perplexus* and its synonyms. However, two such collections in the Kew fungarium yielded sequences that clustered together but were divergent from those of *G. europerplexus*, *G. perplexus* and *G. psittacinus* and may represent a further novel taxon. By contrast, *G. reginae* is morphologically distinct and can usually be recognized in the field by its purplish viscid pileus and relatively stout, flexuose, pale stipe. It is named to commemorate the diamond jubilee of Her Majesty Queen Elizabeth II in 2012 and the 60th anniversary of her coronation in 2013.

## Keywords

Conservation, cryptic species, diamond jubilee, DNA barcoding, fungi, *Gliophorus*, *Glutinosae*, Hygrophoraceae, parrot waxcap, taxonomy, waxcap grasslands

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## Introduction

In Europe, waxcap mushrooms (Hygrophoraceae, *Hygrocybe* s.l.) are conspicuous and often attractive features of nutrient-poor short turf. Hotspots of waxcap taxonomic diversity are usually biocide-free, unfertilised or semi-improved, grazed grasslands or mown lawns with low levels of soil disturbance and long periods of ecological continuity. Such sites are often, but not always, of low botanical interest and this has undoubtedly delayed their recognition as sites of international conservation importance. Historically, therefore, even the best "waxcap grasslands" (using Arnolds' (1980) terminology) have rarely received adequate long-term protection. More recently, waxcap assemblages are increasingly being recognized as useful bioindicators for identifying sites of conservation priority (e.g., Boertmann 1995, 2010, Nitare 2000, McHugh et al. 2001, Griffith et al. 2002, Newton et al. 2003, Evans 2004, Genney et al. 2009). Some species, such as the pink waxcap *H. calyptriformis*, have emerged as flagships for the still nascent practice of targeted conservation of fungi, and waxcaps as a group are becoming mascots for fungal conservation in general.

Waxcaps are regarded as nitrogen-sensitive organisms because fruiting is inhibited by applications of nitrogenous fertilizers (Arnolds 1989). However, their below-ground ecology, in particular their nutritional mode(s), remains unclear despite recent attention by several researchers. Indirect evidence from carbon and nitrogen stable isotope ratios suggests that at least some taxa are biotrophic (Seitzman et al. 2011), and further evidence of a mycorrhiza-like association with plants has been demonstrated recently (Halbwachs et al. unpublished data).

Taxonomic treatments of European waxcaps have recognized from one to seven genera (e.g. Kummer 1871, Orton 1960, Orton and Watling 1969, Kühner 1980, Moser 1983, Kovalenko 1989, Arnolds 1990, Bon 1990, Boertmann 1995, 2010, 2012, Candusso 1997, Krieglsteiner 2001, Bresinsky 2008, Vizzini and Ercole 2012). Molecular phylogenetic analysis indicates that these fungi are not monophyletic and that at least two major phylogenetic clades can be recognized (Babos et al. 2011, Lodge et al. in press). Basidiomata of one group are characterized by vivid yellow, orange and red colours whereas those of the second group lack muscaflavin pigments and are pallid to brown, sometimes showing olive, pink or purple tints (Babos et al. 2011). At least three major groups can be recognized based on hyphal arrangement and compartment lengths within the hymenophoral trama (Boertmann 1995, 2010). These categories are partly supported by phylogenetic evidence (Babos et al. 2011, Lodge et al. in press).

Waxcap identification in Britain and Ireland currently adheres to Boertmann's (1995, 2010) taxonomic concepts. In turn, these concepts are based on basidiomatal macroscopic and microscopic morphology, although it is accepted that some taxa can show overlapping variation. As a result, 51 species (plus eight infraspecific taxa) of *Hygrocybe* s.l. are currently accepted in the online *Checklist of the British and Irish Basidiomycota* (CBIB; http://www.basidiochecklist.info/). However, only a handful of these are individually recognised as species of conservation concern. Five waxcaps were assessed as Vulnerable or Near Threatened in the Great Britain & Isle of Man

unofficial Red Data List (Evans et al. 2006) and only the date waxcap, *H. spadicea*, is currently recognised as a priority species in the UK Biodiversity Action Plan. Not only does morphological identification of waxcaps underpin their current RDL assessment (Evans et al. 2006), it also contributes to the designation of UK sites as Important Fungus Areas (Evans et al. 2001) and Sites of Special Scientific Interest (SSSI). Indeed, waxcaps are currently one of the few groups of fungi for which SSSIs can be designated; any site with at least 18 recorded *Hygrocybe* s.l. species "should be considered for SSSI status" (Genney et al. 2009). Waxcap taxonomy and identification are, therefore, fundamental to their effective conservation.

Recent developments in DNA-based methods of identification ("DNA barcoding") are revolutionizing rapid diagnosis of diversity in mushrooms and other *Fungi* (Dentinger et al. 2011, Schoch et al. 2012). This study is part of a UK-wide initiative that is applying a DNA barcoding approach to waxcaps and revealing surprising levels of unknown diversity. We currently believe that at least 96 species are present in the UK as defined by DNA sequence-based methods (Defra science and research project WC0787). This has involved morphological and molecular analysis, or reanalysis, of 83 fungarium specimens in K whose sequences were published by Brock et al. (2009), 124 newly-sequenced specimens from K, E, and MICH, and more than 600 new field collections mostly from 2011 and 2012.

This paper focuses on our treatment of two unusual waxcaps that, because of their viscid pilei and subregular hymenophoral tramal hyphae, are assigned to the segregate genus *Gliophorus*. They share some morphological characters with the parrot waxcap *G. psittacinus*, which encompasses a wide range of basidiomatal pigmentation based on current concepts. Numerous colour forms can be recognised (Boertmann 2001) but, partly because this character is known to be influenced by ageing and weather conditions, formal taxonomic resolution into recognisable segregate species has proved more challenging. Four varieties are listed in Index Fungorum (http://www.indexfungorum.org). Our unusual collections lacked green pigments and one group matched the type description of *Hygrophorus perplexus* A.H. Sm. & Hesler, a North American species. This is recorded in Europe where, as one of the few accepted parrot waxcap segregates, it is currently recognised as *Hygrocybe psittacina* var. *perplexa* (Boertmann 2012). Molecular analysis, including sequences derived from type specimens of *Hygrophorus perplexus*, collections filed as *H. psittacina* var. *perplexa* in K and downloaded from GenBank labelled as *H. psittacina*, confirmed the presence of two new species lacking green pigments, which we describe here.

## Methods

### Taxon and specimen sampling

A total of 20 collections corresponding to the *G. psittacinus* complex were sequenced and morphologically examined in the current study. These comprised 12 recent UK field collections now in K, four existing K collections from UK and Jersey and four US type collec-

tions in MICH. Table 1 shows the relevant collection details. Further details for specimens of *G. europerplexus* and *G. reginae* are provided in the taxonomic treatment below. Geographical coordinates of collections were converted from Ordnance Survey National Grid References, based on the OSGB36 datum, to latitude and longitude (WGS84 datum).

## Morphological analysis

Spore measurements are rounded to the nearest half micron and preceded by associated data in square brackets. For example, [60, K(M)181128*, K(M)181129] would indicate that 60 spores in total were measured either in water from prints (*G. reginae*) or in Melzer's reagent from lamellar squashes (*G. europerplexus*) from the collections K(M)181128 and K(M)181129. Collections sequenced during this study, such as K(M)181128 in this example, are denoted throughout by *. Measurements of basidia and other hyphal elements are rounded to the nearest micron. Colours given in parentheses refer to those shown in a standard mycological identification chart (Anon 1969).

## DNA extraction and sequencing

DNA was extracted using either an enzymatic digestion-glass fiber filtration protocol in 96-well plate format with a vacuum-manifold or the Whatman FTA® card method described in Dentinger et al. (2010). Full and partial nuclear ribosomal internal transcribed spacer regions (ITS) were amplified and sequenced with primers ITS1F/ITS3 and ITS2/ITS4 (White et al. 1990, Gardes and Bruns 1993) or with primers ITS8F and ITS6R (Dentinger et al. 2010) following the cycling conditions in Dentinger et al. (2010). PCR products were visualized by UV fluorescence after running out 2 μL PCR products in a 1% agarose gel containing 0.005% ethidium bromide. Prior to sequencing, positive PCRs were cleaned by adding 0.5 μL ExoSAP-IT to every 2.5 μL PCR reaction mix and incubating this mix for 15 min at 37 C followed by 15 min at 80 C. Unidirectional dye-terminator sequencing used the ABI BigDye kit (Foster City, CA) following the manufacturer's instructions except reducing the total reaction volume to 5 μL. Sequencing reactions were cleaned using ethanol precipitation and resuspended in distilled water before loading into an ABI PRISM 3730 DNA Analyzer in the Jodrell Laboratory, Royal Botanic Gardens, Kew. Complementary unidirectional reads were aligned and edited using Sequencher4.2 (GeneCodes, Ann Arbor, MI). All new sequences have been deposited in the International Nucleotide Sequence Database (Accession numbers: KF218257–KF218275).

## Phylogenetic analysis

Six additional sequences labelled as *H. psittacina* (Brock et al. 2009, Babos et al. 2011) were downloaded from GenBank and combined with our dataset (Table 1). The se-

**Table 1.** Collection and voucher information for the specimens used in this study.

| GenBank Accession No. | Taxon | Fungarium Accession No. | Collection code/ seq. literature ref. | Source | Notes |
|---|---|---|---|---|---|
| KF218268 | *Gliophorus europerplexus* | K(M)181241 | E.J.M.Arnolds WX359 | UK, Wales, Merionethshire | |
| KF218266 | *Gliophorus europerplexus* | K(M)181245 | D.J.Harries DJH064A WX663 | UK, Wales, Pembrokeshire | |
| KF218267 | *Gliophorus europerplexus* | K(M)181246 | D.J.Harries DJH064B WX664 | UK, Wales, Pembrokeshire | Holotype |
| KF218272 | *Gliophorus perplexus* | MICH10924 | A.H.Smith 21491 | USA, Michigan, Cheboygan Co. | *Hygrophorus perplexus* holotype, part |
| KF218270 | *Gliophorus perplexus* | MICH45363 | T.E.Brooks 1098 | USA, Michigan, Cheboygan Co. | *Hygrophorus perplexus* paratype |
| KF218271 | *Gliophorus perplexus* | MICH45364 | T.E.Brooks 1099 | USA, Michigan, Cheboygan Co. | *Hygrophorus perplexus* paratype |
| KF218274 | *Gliophorus perplexus* aff. | K(M)121495 | E.W.Brown | Channel Islands, Jersey | as *Hygrocybe psittacina* var. *perplexa* |
| KF218273 | *Gliophorus perplexus* aff. | K(M)166625 | N.W.Legon | UK, England, South Somerset | as *Hygrocybe psittacina* var. *perplexa* |
| KF218269 | *Gliophorus perplexus* | MICH45365 | A.H.Smith 34029 | USA, Michigan, Chippewa Co. | Hygrophorus perplexus paratype |
| EU784339 | *Gliophorus psittacinus* | K(M)127070 | Brock et al. 2009 | UK, N. Ireland, Derry | |
| EU784340 | *Gliophorus psittacinus* | K(M)127194 | Brock et al. 2009 | UK, N. Ireland, Tyrone | |
| EU784341 | *Gliophorus psittacinus* | K(M)90029 | Brock et al. 2009 | UK, England, Buckinghamshire | |
| EU784342 | *Gliophorus psittacinus* | K(M)90674 | Brock et al. 2009 | UK, England, East Sussex | |
| FM208875 | *Gliophorus psittacinus* | | Babos et al. 2011 | Hungary, Kétvölgy | |
| FM208895 | *Gliophorus psittacinus* | | Babos et al. 2011 | Hungary, Apátistvánfalva | |
| KF218259 | *Gliophorus reginae* | K(M)156265 | R.Winnall | UK, England, Worcestershire | Holotype |
| KF218258 | *Gliophorus reginae* | K(M)181115 | R.D.Foster WCS15 WX115 | UK, England, Derbyshire | |
| KF218260 | *Gliophorus reginae* | K(M)181116 | R.D.Foster WCS26 WX126 | UK, England, Derbyshire | |
| KF218263 | *Gliophorus reginae* | K(M)181117 | R.Winnall WX459 | UK, England, Worcestershire | |
| KF218265 | *Gliophorus reginae* | K(M)181124 | J.E.Hodges DJH055 WX535 | UK, Wales, Pembrokeshire | |
| KF218264 | *Gliophorus reginae* | K(M)181126 | R.Winnall WX673 | UK, England, Worcestershire | |
| KF218275 | *Gliophorus reginae* | K(M)181127 | R.Winnall & A.M.Ainsworth WX694 | UK, England, Worcestershire | |
| KF218257 | *Gliophorus reginae* | K(M)181128 | R.Winnall & A.M.Ainsworth WX695 | UK, England, Worcestershire | |
| KF245883 | *Gliophorus reginae* | K(M)181129 | R.Winnall & A.M.Ainsworth WX696 | UK, England, Worcestershire | |
| KF218262 | *Gliophorus reginae* | K(M)181227 | R.Winnall WX461 | UK, England, Worcestershire | |
| KF218261 | *Gliophorus reginae* | K(M)41524 | C.Lovatt | UK, England, Staffordshire | |

quences were trimmed to minimize uneven ends across the dataset and aligned using the RNA structure-based algorithm Q-INS-i implemented in MAFFT v7.023b (Katoh et al. 2002, Katoh and Toh 2008, Katoh and Standley 2013). Phylogenetic analysis under the maximum likelihood criterion was performed using the Pthreads-parallelized version of RAxML v7.0.3 (Stamatakis 2006, Ott et al. 2007) with a GTR-GAMMA model. Branch support was assessed using nonparametric bootstrapping with the "thorough" option and 1000 replicates. The final alignment and phylogenetic tree are available from TreeBase (#14384; http://purl.org/phylo/treebase/phylows/study/TB2:S14384 ).

## Results

The full ITS region was amplified and sequenced for 14 specimens. Only the ITS1 region was sequenced for all specimens from MICH, while only the ITS2 region was sequenced for K(M)181124. The ITS1 and ITS2 regions were amplified and sequenced separately for K(M)181245, and the two non-overlapping regions were concatenated and separated by 66 gaps corresponding to the 5.8S ribosomal subunit in the final alignment. Phylogenetic analysis resulted in a highly resolved tree with most nodes receiving strong bootstrap support (Fig. 1). Both *G. psittacinus* and *G. perplexus* were found to be polyphyletic. Two distinct clades were strongly supported (100%): 1) *G. reginae* (99%) and a subclade (73%) consisting of *G. europerplexus* (99%) and a single sequence from a paratype specimen of *G. perplexus*, and 2) all other sequences, including three *G. psittacinus* clades (87%, 100%, 100%) comprising the GenBank sequences, one *G.* aff. *perplexus* clade (100%), and one clade composed of sequences from the holotype and two paratype specimens of *G. perplexus* (100%).

### Taxonomic treatment

***Gliophorus reginae*** **Dentinger, A.M.Ainsw., & P.F.Cannon, sp. nov.**
Registration Identifier: IndexFungorum IF 550184
http://species-id.net/wiki/Gliophorus_reginae
Figures 2, 3, 6

**Holotype.** UNITED KINGDOM. England. Worcestershire (vice county 37): Bewdley, Willow Bank, 52°21.46'N, 2°22.42'W (Nat. Grid Ref. SO746733), 24 Jan 2008, R.Winnall (K(M)156265)

**Description.** Pileus 15–55 mm diam., hemispherical to broadly conical or campanulate, initially with incurved margin, becoming applanate, often retaining broad umbo and irregular, lobed outline with indentations, folds and pleats, sometimes becoming radially furrowed, or split and flared, margin faintly to strongly translucently striate to half-way and becoming reflexed to highly revolute, viscid with gelatinous

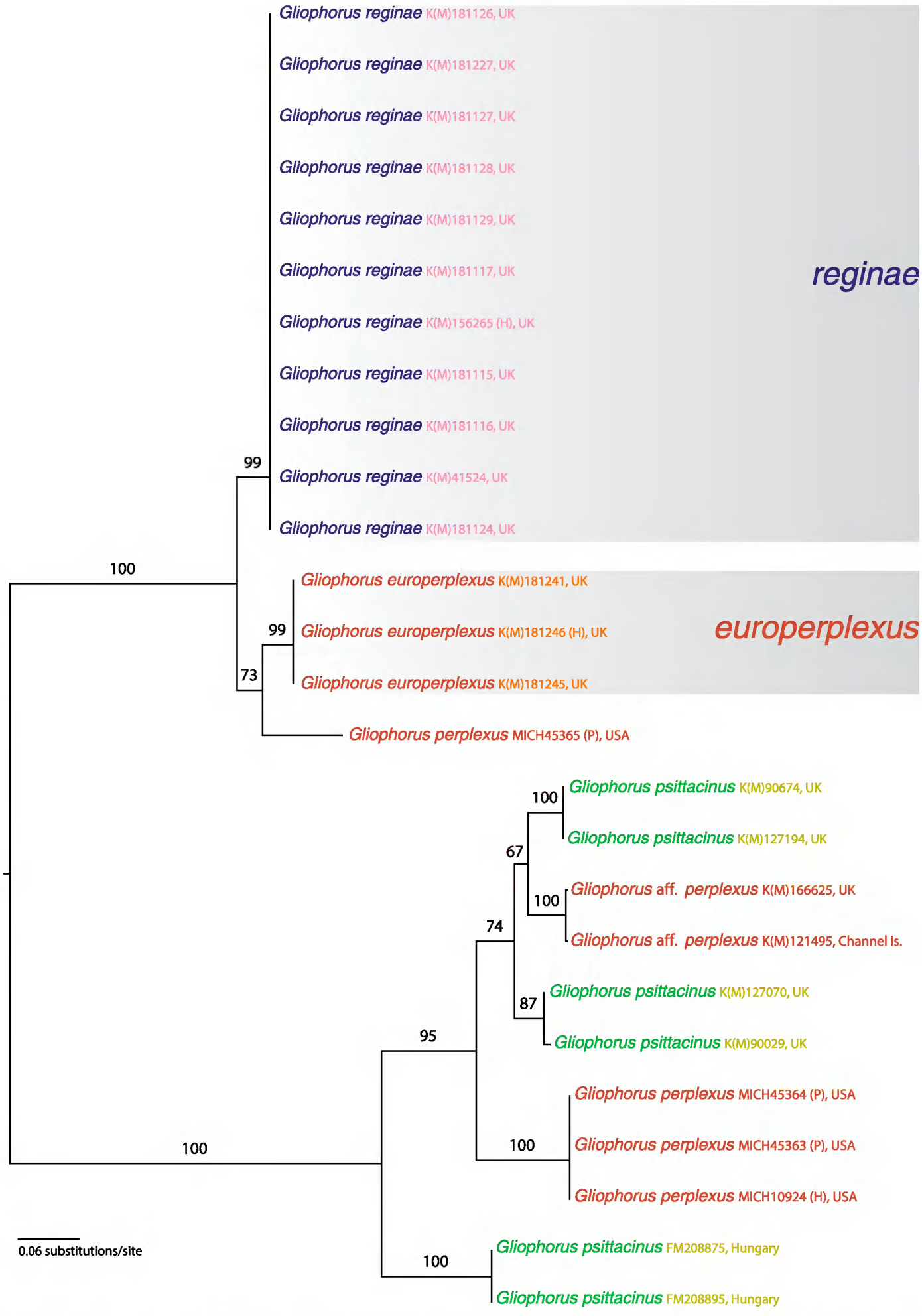


**Figure 1.** Maximum likelihood phylogram using full and partial nuclear ribosomal internal transcribed spacers (ITS) sequences. Numbers above branches are nonparametric bootstrap values. Tree is arbitrarily rooted at the midpoint. Two well-supported terminal clades representing the new species *G. reginae* and *G. europerplexus* are superimposed over light grey boxes. Species names for specimens from which sequences were derived are followed by fungarium or INSD accession number, and geographic location. Notations (H) and (P) indicate specimens used were holotypes or paratypes, respectively.

**Figure 2.** Basidiomata of *G. reginae* showing pileal colour range: **A–D** purple, **E–F** pink and **G–H** reddish brown, scale bars represent 20 mm. Photographs **C–F** taken *in situ* at the type locality. **A, B** K(M)181115* photographed by R.D. Foster. **C** K(M)181117* and **D** K(M)181123 photographed by R. Winnall. **E** (left), **F** K(M)181128* and **E** (right) K(M)181127* photographed by A.M.A. **G, H** K(M)181124* photographed by D.J. Harries.

pellicle, sometimes with minutely rugose texture, at first usually dull violet purple (vinaceous grey to purple) with areas of pink, darker red or red-brown tones (rose, blood red to rusty tawny), sometimes more brownish (purplish date to dark brick), becoming paler and pinkish especially around margin which can also develop yellow (luteous) or yellow-brown (fulvous) tints, hygrophanous, dried pilei characteristically pale orange (saffron) flushed pink (rose). Lamellae ventricose, mostly narrowly adnate with some free, sinuate or broadly adnate elements, intervenose, concolorous with pileus near pileal attachment, becoming paler towards free edge, sometimes with yellow (luteous) or orange (saffron) tints. Stipe 15–70 × 5–15 mm, relatively stout, sometimes tapering upwards from the clavate base, hollow, often flexuose or tortuous, compressed or grooved, viscid but usually slightly less so than pileus, white often apically tinged with pileal colour and basally yellow (luteous) to pale orange (saffron) or becoming so, sometimes with purplish (vinaceous grey) blotches if frosted. Outer tissues of context concolorous with adjacent external surfaces, inner tissues paler. Dried lamellar trama (lens) often conspicuously dark pink (coral), contrasting with paler subhymenium and lamellar surfaces. Green pigments entirely absent. Without distinctive taste or smell. Spores [120, K(M)181126*, K(M)181127*, K(M)181128*, K(M)181129*] 6.0–8.5(–9.0) × 4.0–5.5(–6.0) µm, per-basidioma mean values 7.0–7.5 × 5.0 µm, Q = 1.2–2.0, mean 1.5, short-ellipsoidal to ellipsoidal, not constricted. Basidia predominantly 4-spored, clavate, relatively long and slender with long attenuated base, (37–)40–63(–67) × 6–10 µm excluding sterigmatal length (4.0–8.0 µm). Clamp connections on basidia, within lamellar trama and pileipellis often with conspicuously looped hook cells (medallion clamps). Lamellar trama subregular with some interwoven elements, compartments 24–183 × 4–24 µm. Stipitipellis and pileipellis are ixotrichoderms.

**Distribution.** Known from a cemetery in West Wales (Pembrokeshire) and fields in central England (Worcestershire, Staffordshire and Derbyshire). The earliest known collection was made by C. Lovatt in Staffordshire in 1996 who noted that she had recorded similar specimens in 1994. It has fruited on private land at Willow Bank (Worcestershire) almost every year from 2000 onwards and recorded there in five discrete fruiting patches in a single field of ca. 0.8 ha.

**Ecology.** In unimproved short (grazed or mown) acid-neutral rough pasture or other grassland. This species is often a relatively late fruiter and can continue producing basidiomata in January, long after other waxcaps have finished.

**Etymology.** Latin *reginae* meaning "of a queen", named for the royal purple colour of the basidiomata and to celebrate the diamond jubilee of Her Majesty Queen Elizabeth II in 2012 and the 60$^{th}$ anniversary of her coronation in 2013.

**Conservation status.** Collectors noted that although basidiomata of this species resembled *G. psittacinus*, some characters such as pileal colour and radial splitting, were more characteristic of *H. calyptriformis.* Furthermore, dried collections of the latter and *G. reginae* often attained a similar reddish-coral tint in the fungarium that was distinct from the pale saffron of *G. psittacinus*. This similarity facilitated a rapid search of the British *G. psittacinus* collections at Kew, but no additional *G. reginae* specimens were

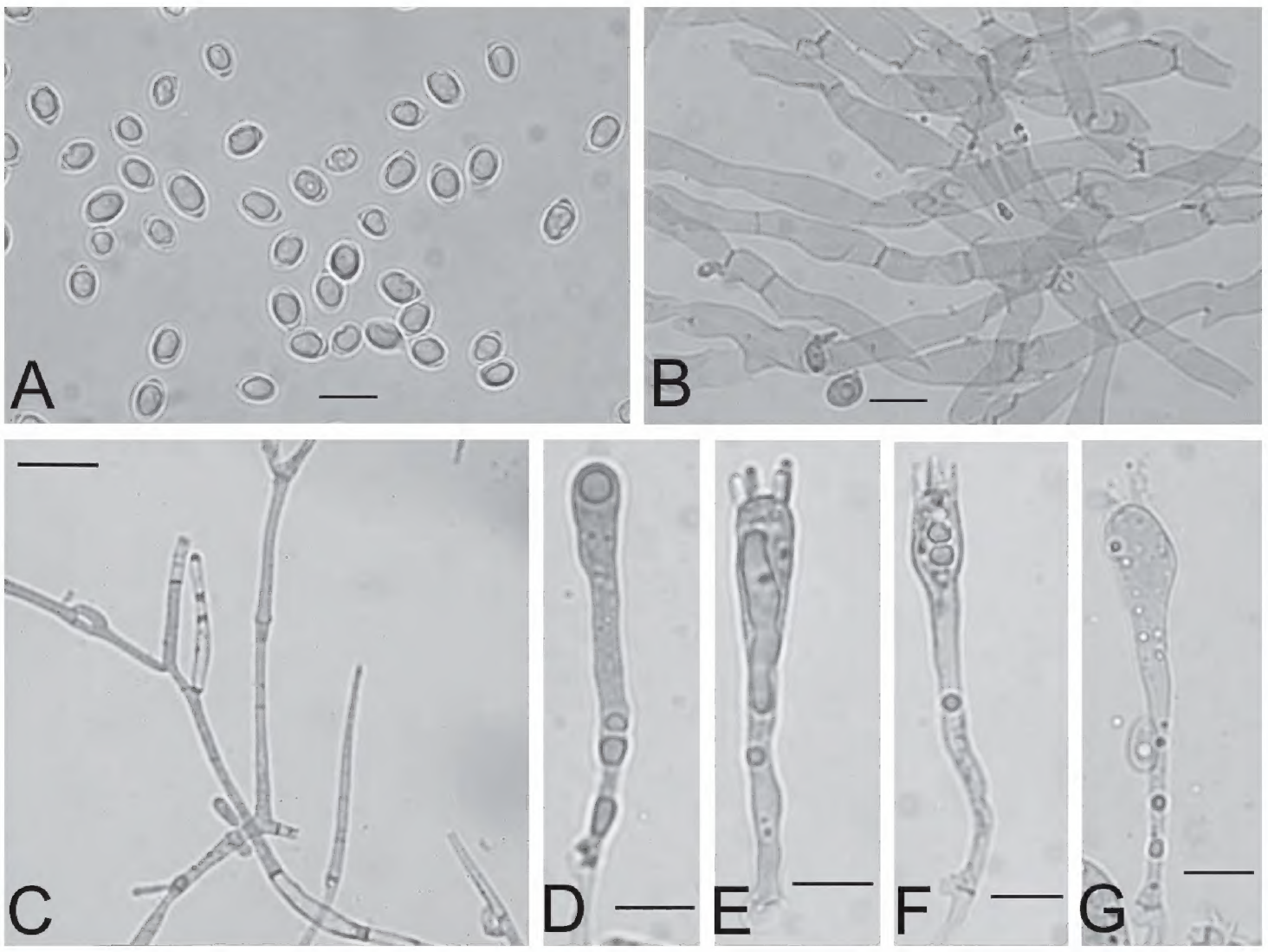


**Figure 3.** Microscopic characters of *G. reginae* collected from the type locality, **B–G** mounted in Congo Red, scale bars represent 10 µm. **A** Spores from water mount of spore print K(M)181127* **B** Subregular lamellar trama from squash mount K(M)181129* **C** Pileipellis hyphae showing medallion clamp connections K(M)181127* **D–F** Basidial developmental series K(M)181129* **G** Basidium K(M)181127*.

discovered. This suggests that it is genuinely rare in Britain and Endangered (EN D, <250 mature individuals) might be the current regional conservation assessment following IUCN guidelines, categories and criteria (IUCN 2012a, b, 2013). However, we think that this species is so poorly known at present that it should be assessed as Data Deficient pending further survey work.

**Other specimens examined.** United Kingdom. England. Derbyshire (vice county 57): Edale, Lower Hollins Farm, 53°21.84'N, 1°47.96'W (Nat. Grid Ref. SK134852), 5 Oct 2010, R.D.Foster WCS8 WX108 (K(M)181114). Ibid. 17 Oct 2010, R.D.Foster WCS15 WX115 (K(M)181115*). Woodlands Valley, Rowlee Bridge Fields, 53°23.99'N, 1°46.60'W (Nat. Grid Ref. SK149892), 9 Nov 2010, R.D.Foster WCS26 WX126 (K(M)181116*). Staffordshire (vice county 39): Danebridge (near), 53°10.25'N, 2°3.99'W (Nat. Grid Ref. SJ956637), 22 Oct 1996, C.Lovatt (K(M)41524*, sub *H. psittacina*). Worcestershire (vice county 37): Bewdley, Bowcastle Farm cherry orchard, 52°22.38'N, 2°20.40'W (Nat. Grid Ref. SO769750), 3 Nov 2004, R.Winnall WX461 (K(M)181227*). Bewdley, Willow Bank, 52°21.46'N, 2°22.42'W (Nat. Grid Ref. SO746733), 4 Nov 2001, R.Winnall (K(M)92058, sub *H.* cf. *psittacina*). Ibid. 11 Nov 2004, R.Winnall WX459 (K(M)181117*). Ibid.

15 Dec 2004, R.Winnall WX460 (K(M)181123). Ibid. 18 Oct 2012, R.Winnall WX673 (K(M)181126*). Ibid. 15 Jan 2013, R.Winnall & A.M.Ainsworth WX694 (K(M)181127*), WX695 (K(M)181128*), WX696 (K(M)181129*). Wales. Pembrokeshire (vice county 45): Fishguard Cemetery, 51°59.30'N, 4°57.64'W (Nat. Grid Ref. SM96803634), 11 Nov 2011, J.E.Hodges DJH055 WX535 (K(M)181124*).

***Gliophorus europerplexus*** **Dentinger, A.M.Ainsw., & P.F.Cannon, sp. nov.**
Registration Identifier: IndexFungorum IF 550185
http://species-id.net/wiki/Gliophorus_europerplexus
Figures 4–6

**Holotype.** UNITED KINGDOM. Wales. Pembrokeshire (vice county 45): Hundleton, Somerton Farm, 51°39.88'N, 4°59.54'W (Nat. Grid Ref. SM931004),11 Oct 2012, D.J.Harries DJH064B WX664 (K(M)181246)

**Description.** Pileus 10–25 mm diam., hemispherical to conical or campanulate, sometimes with incurved margin, becoming plano-convex or remaining broadly conical, often umbonate, sometimes with irregular, lobed outline, margin faintly to strongly translucently striate to half-way, viscid or at least very lubricous, sometimes partially flared, at first usually pink-brown to orange-brown (brick, rusty tawny to fulvous), margin paler sometimes with orange (sienna to apricot) tints, hygrophanous, dried pilei dull orange (saffron to rust). Lamellae ventricose, mostly narrowly to broadly adnate with some slightly decurrent elements, intervenose, concolorous with pileus near pileal attachment, becoming paler towards free edge. Stipe 12–60 × 2–8 mm, cylindrical or compressed, sometimes with clavate base, hollow, sometimes flexuose, viscid but usually slightly less so than pileus, apically concolorous with pileus, paler below, sometimes basally tinted orange (apricot). Dried lamellar trama (lens) often darker than subhymenium and lamellar surfaces. Green pigments entirely absent. Without distinctive taste or smell, although one specimen [K(M)181241*] was noted to have a faint rubbery smell reminiscent of *G. laetus*. Spores [70, K(M)181241*, K(M)181245*, K(M)181246*] (6.5–)7.0–9.0 × (4.0–)4.5–5.5(–6.0) µm, per-basidioma mean values 7.5–8.0 × 5.0 µm, Q = 1.3–1.8, mean 1.6, short-ellipsoidal to ellipsoidal, not constricted. Basidia predominantly 4-spored, clavate with attenuated base, (30–)34–57 × 5–9 µm excluding sterigmatal length (3.0–7.0 µm). Clamp connections on basidia, within lamellar trama and pileipellis usually normal, occasionally with conspicuously looped hook cells (medallion clamps). Lamellar trama subregular, compartments 20–120 × 4–21 µm. Stipitipellis and pileipellis are ixotrichoderms.

**Distribution.** Identified from two sites in west Wales (Merionethshire and Pembrokeshire) supported by DNA sequence data. Fruiting was probably observed at the type locality by D.J. Harries on 6 August 2009 but no material was kept.

**Ecology.** In unimproved short acid-neutral rough pasture in Merionethshire and found fruiting on bare soil near mosses on an almost vertical south-facing earth bank on farmland in Pembrokeshire.

**Figure 4.** Basidiomata of *Gliophorus europerplexus*, scale bars represent 10 mm. Photographs **A–D** by D.J. Harries taken *in situ* at, or of collections from, the type locality, and **E** by B.T.M.D. **A, B** K(M)181245* **C, D** K(M)181246* holotype **E** K(M)181241*.

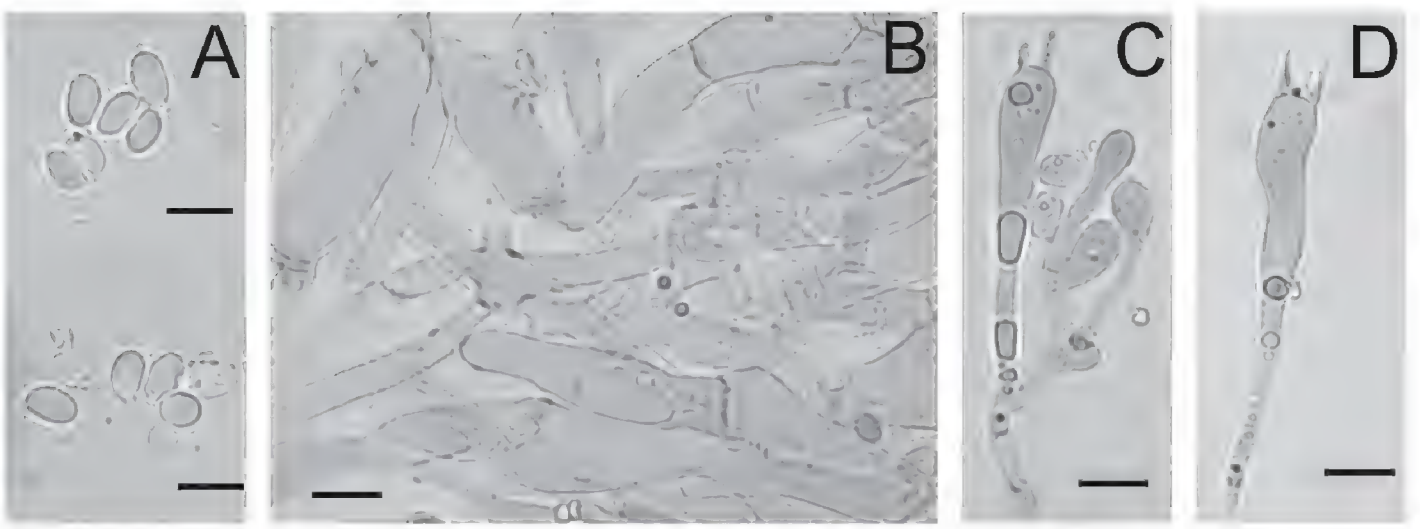


**Figure 5.** Microscopic characters of *G. europerplexus*, **A** mounted in Melzer's Reagent and **B–D** (holotype) in Congo Red, scale bars represent 10 μm. **A** Spores from lamellar squash K(M)181241* **B** Subregular lamellar trama from squash mount K(M)181246* **C–D** Basidia K(M)181246*.

**Etymology.** Named to distinguish this European taxon from the morphologically similar *Hygrophorus perplexus* A.H.Smith & Hesler, a species with North American type material.

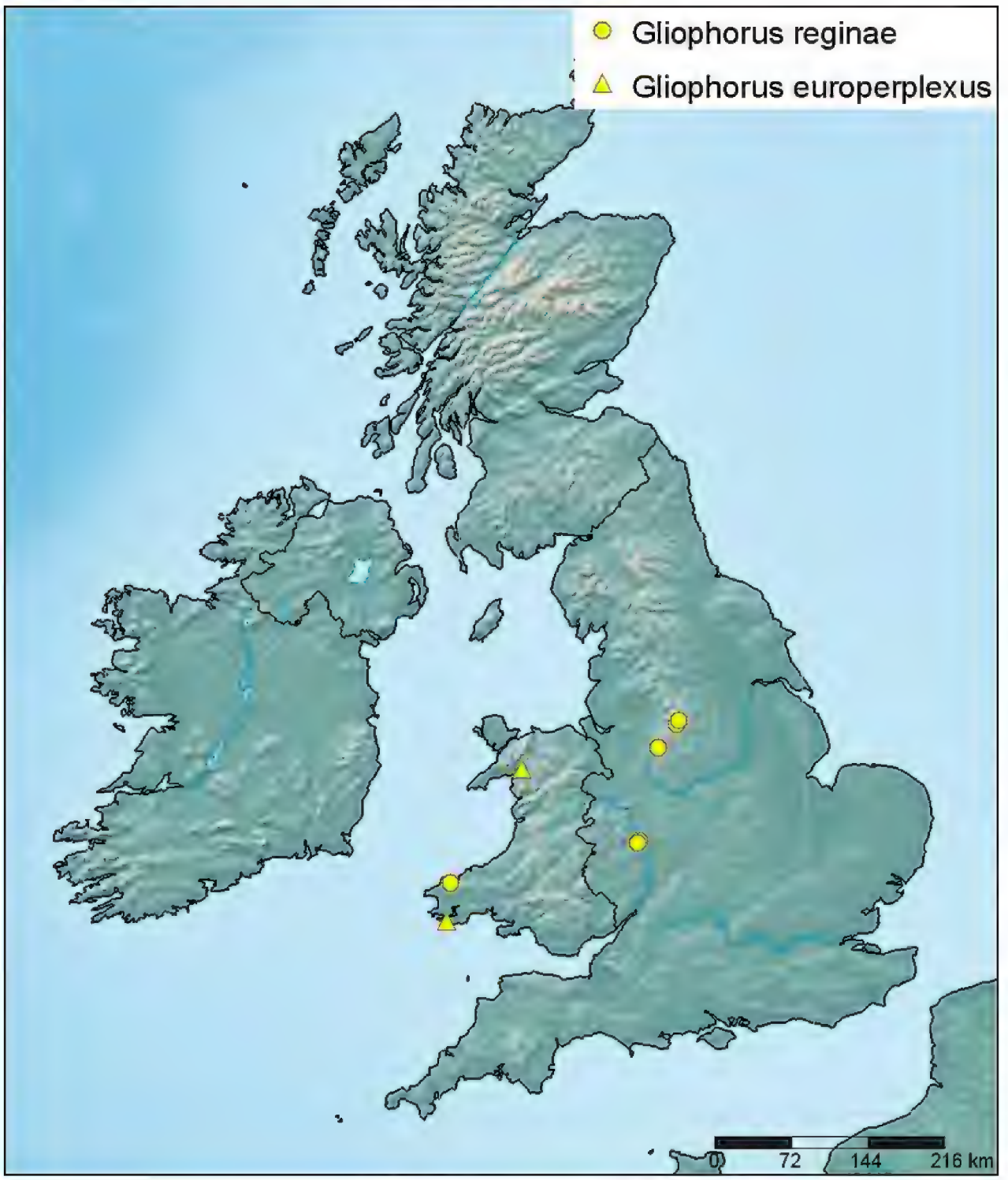


**Figure 6.** Distribution of *G. reginae* (●) and *G. europerplexus* (▲) in Britain based on sequenced collections and plotted using SimpleMappr (Shorthouse 2010).

**Conservation status.** Initially, it seemed likely that historic British collections assigned to *H. psittacina* var. *perplexa* would be redetermined as *G. europerplexus* following DNA sequencing. However, two specimens filed in K under the former name yielded distinct ITS sequences (Fig.1). Therefore, the distribution of *G. europerplexus* is currently unknown and it should be assessed as Data Deficient.

**Other specimens examined.** United Kingdom. Wales. Merionethshire (vice county 48): Croesor, Cnicht, 52°58.34'N, 4°0.22'W (Nat. Grid Ref. SH64 but estimated to be SH655435 for conversion to latitude and longitude), 11 Oct 2011, E.J.M.Arnolds WX359 (K(M)181241*). Pembrokeshire (vice county 45): Hundleton, Somerton Farm, 51°39.88'N, 4°59.54'W (Nat. Grid Ref. SM931004), 19 Aug 2012, D.J.Harries DJH064A WX663 (K(M)181245*; immature).

## Discussion

The traditional *Gliophorus* (=*Hygrocybe*) *psittacinus* species concept is relatively broad (Boertmann 2001), mainly due to difficulties in defining unambiguous morphological discontinuities in basidiomatal characters. Our ITS sequence analysis revealed a clade comprising two terminal clusters, *G. reginae* and *G. europerplexus*, together with a singleton, a paratype of *H. perplexus*, that is clearly divergent from that comprising the currently-accepted European taxon *G. psittacinus* and the N. American *G. perplexus* (Fig. 1). It is clear that the name *G. psittacinus* currently represents a species complex and further work is required to characterise and describe the component taxa. We have assigned our two new species to the segregate waxcap genus *Gliophorus* Herink based on recent supporting molecular phylogenetic evidence (Babos et al. 2011, Lodge et al. in press).

One of the novel taxa, *G. reginae*, is recognisable in the field having a relatively stout stipe, sometimes yellowing at the base, and distinctive deep purple or reddish-brown pileus. Our field observations suggest that a colour form of this might be shown in Boertmann's photograph of Danish specimen DB 2000/33 taken at Lysnet, E. Jylland, on 17 Oct. 2000 (Boertmann 2001 fig. 4, 2010 p.91). The remaining currently-accepted European taxon in the parrot waxcap group is *H. psittacina* var. *sciophanoides* (Boertmann 2010, 2012). This species was originally described as *Hygrophorus sciophanoides* by Rea (1922) based on a painting of an English specimen found in 1909 in Derbyshire designated as Rea 937, but no type specimen is preserved at Kew. Rea's "uncommon" fungus had a rosy pink striate pileus 1-3 cm diam. with pale pink lamellae, a concolorous stipe 2–5 cm × 2–3 mm and flesh described as pale yellow becoming white. Although Rea did not use the word "viscid" in his description, nevertheless he synonymised Cooke's (1889) concept of *Hygrophorus sciophanus*, a slightly viscid-pileate species with decurrent gills, with *H. sciophanoides*. Cooke (1889) quoted a description of two Scottish specimens, one pale and sterile and the other darker and yielding "very pale clay-coloured" spores from *Notices of British Fungi No. 1560* (Berkeley and Broome 1876). The latter authors also recorded some small Welsh specimens in *Notices of British Fungi No. 1885*, again noting the existence of light and dark forms (Berkeley and Broome 1881). Cooke's (1886–1888) six illustrations (No. 905 Plate 937A) of English material from Kendal resemble the specimen depicted by Rea. *G. reginae* can be similarly pink and striate (Fig. 2F), but Rea and Cooke both described and illustrated basidiomata that were strikingly more slender than those of *G. reginae*. This together with the lack of type material of *H. sciophanoides* and the existence of other colour forms of the *H. psittacina* complex that can develop pink tints with age, leads us to conclude that *H. sciophanoides* should be regarded as a *nomen dubium*. Our attempts to sequence Welsh material collected in 1950 (K(M) 69657) and determined by Pearson as *H. sciophanoides* were unsuccessful.

In Europe, *Hygrophorus sciophanus* (Fr.) Fr. is currently regarded as a synonym of *H. psittacina* var. *perplexa* with *Hygrophorus perplexus* A.H. Sm. & Hesler as basionym. By contrast, Hesler and Smith (1963) argued that their taxon had a very similar lamellar attachment, "never decurrent", to that of *Hygrophorus psittacinus*, a character

that distinguished it from *H. sciophanus*. Indeed Fries distinguished the lamellar attachment of *Agaricus psittacinus*, described as "adnatis", from that of *A. sciophanus*, "decurrentibus" (Fries 1821) and, later, of *H. sciophanus*, "subdecurrentibus" (Fries 1836–1838). Rea (1922), on the other hand, described the attachment in *H. sciophanus* as "attenuato-adnate", as shown in Rea 936, a painting of a French collection, and he cited an illustration approved by Fries. The latter painting (Fries 1877–1884, Plate 167.1) appears to bear out Rea's description, but in the same volume (p. 66), Fries used the word "decurrentibus" in the diagnosis and commented that the illustration showed "lamellarum insertio minus typica". The original concept of *H. sciophanus* thus is unclear and various interpretations exist in the literature. Three collections originally filed as *Hygrophorus sciophanus* preserved in K were sequenced and determined to be highly divergent from the *Gliophorus* sequences in our dataset, belonging instead to *Hygrocybe* sensu stricto (data not shown). In our view, *H. sciophanus* should be regarded as a *nomen dubium*.

Our analysis showed that the ITS sequence derived from the holotype specimen of *H. perplexus* is certainly distinct from the second of our new species, *G. europerplexus*. Two specimens identified as *H. psittacina* var. *perplexa* (Table 1) collected in 2003 and 2008 were also sequenced, but they are phylogenetically distinct, forming a clade near to *G. psittacinus* (Fig. 1) and may represent a further novel taxon. The single anomalous sequence from a paratype of *H. perplexus*, which comes near *G. europerplexus* in our analysis, reveals additional cryptic diversity within this species complex in North America and highlights the difficulty in correctly naming waxcap species using morphology alone. Attempts should be made, therefore, to sequence additional European and North American specimens currently filed as *G. perplexus*, *Hygrophorus perplexus*, *Hygrocybe perplexa* and *H. psittacina* var. *perplexa* to gain a better understanding of the distribution of *G. europerplexus* and other emerging segregate taxa.

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